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Nitric Oxide and Major Depressive Disorder

by

Wendy Chrapko



A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of
the

requirements for the degree of Master of Science

Department of Psychiatry

Edmonton, Alberta
Fall 2003



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ABSTRACT

There is an established link between major depressive disorder (MD) and cardiovascular disease (CVD) however the mechanism linking MD and CVD is yet undetermined. The purpose of this thesis is to investigate nitric oxide (NO) as a potential mechanism due to the importance of NO in the cardiovascular system. NO production is measured before and after treatment with paroxetine. Plasma NO metabolite (NOx) levels were measured by chemiluminescence. Platelet NO synthase (NOS) activity was examined by the citrulline assay. Levels of both plasma NOx and platelet NOS activity were significantly lower in subjects with MD versus healthy controls (HCs) prior to treatment. Throughout treatment plasma NOx levels increased in both HCs and MD patients, however platelet NOS activity decreased in HCs while no change was evidenced in MD patients. These data suggest that decreased NO production, a potential contributor to increased cardiovascular risk, is modified by administration of the paroxetine.

DEDICATION

This is dedicated to my amazing parents, Bill and Helen. Thank you for your limitless love, never-ending support and most importantly your belief in me. I love you both so much.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Jean-Michel Le Mellédo for his support and encouragement, especially over the last few months. I would also like to thank the co-authors on the manuscripts in this thesis, for their input, revisions and assistance. Specifically thank you to Nathalie Lara, Paul Jurasz, Marek Radomski, Stephen Archer, Steve Newman and Glen Baker. Finally I want to thank Dr. Andy Greenshaw, who has taught me so much about science, psychiatry and believing in myself and doing the right thing.

I also want to thank all my friends, too many to list, for being there for me. Specifically, I want to thank Emily Bell for always making me smile, keeping my life in perspective and keeping my closet full. Jocelyn Lock, thank you for listening, understanding and never thinking I am crazy. Brad Wuetherick, thank you for your hugs and support and sharing your food. Grant Gay, thank you for being there to listen, always offering a solution to any problem and keeping my "debate" skills sharp. Finally, Doug Griffiths, thank you for your support, for taking my mind off it all when necessary yet knowing to leave me alone when I needed my mind on it all.

And finally thanks to the fabulous people at the Clinical Investigation Unit, Lori Zuk, Laurentina Tolvay, Stephen Culver, Cinnamin Lewandowski and Rosann Lastiwska, for being so flexible and understanding and always being there to take blood!

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BH4	Tetrahydrobiopterin
BMI	Body Mass Index
°C	Degree Celsius
Ca ²⁺	Calcium
CHD	Coronary Heart Disease
CPR	Cytochrome P450 reductase
CVD	Cardiovascular Disease
DBP	Diastolic Blood Pressure
df	Degrees of Freedom
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders – 4 th Ed.
EDRF	Endothelial Derived Relaxing Factor
EDTA	Ethylenediaminetetraacetate
EGTA	Ethyleneglycolbis-(aminoethylether)tetra-acetate
eNOS	Endothelial Nitric Oxide Synthase
FAD	Flavine Adenine Dinucleotide
FMN	Flavin Mononucleotide
HCl	Hydrochloric Acid
HCs	Health Controls
HDL	High Density Lipoprotein
iNOS	Inducible Nitric Oxide Synthase
kcal/kg/day	Kilocalorie per kilogram per day
kg/m ²	Kilogram per metre squared
LDL	Low Density Lipoprotein
L-NMMA	N(G)-mono-methyl-L-arginine
MD	Major Depressive Disorder
mg/day	Milligram per Day
mg/L	Milligram per litre

min	Minute
mL	Millilitre
μ M	Micromolar
μ m	Micromole
mm	Millimetre
mm/Hg	Millimetres of Mercury
μ mol/L	Micromole
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
nM	Nanomolar
nm	Nanometre
nmol/L	Nanomole per Litre
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NO _x	Nitric Oxide Metabolites
p	Probability
PGI ₂	Prostacyclin
pmol/min/mg protein	Picomole per minute per milligram protein
RPM	Rotations Per Minute
SBP	Systolic Blood Pressure
SEM	Standard Error of Mean
SSRIs	Selective Serotonin Re-uptake Inhibitors

CHAPTER 1

General Introduction: Major Depressive Disorder, Cardiovascular Disease and Nitric Oxide

1.1 INTRODUCTION

Cardiovascular disease (CVD) is the umbrella term used to describe a broad group of conditions, which includes coronary heart disease (CHD) and cerebrovascular disease. In CHD the function of the heart muscle is impaired by reduced blood supply, most often due to atherosclerosis, and is manifested as angina, acute myocardial infarction, chronic ischemic heart disease, or sudden death. Other CVD include arrhythmias, hypertension, valvular heart disease, and peripheral vascular disease.

The conventional risk factors for CVD are classified as non-modifiable or modifiable. The non-modifiable risk factors are family history of CVD, age, and male-sex. The modifiable risk factors are, for example, smoking, hypertension, physical inactivity, high blood cholesterol levels, diabetes, and obesity. Conventional risk factors, however, explain only 40% of the incidence of CVD. One of the unconventional risk factors suggested to help account for the rest of the risk is major depressive disorder (MD).

MD, as defined in the Diagnostic and Statistical Manual for Mental Disorders, 4th edition¹, is characterized by depressed mood and/or loss of interest and pleasure persisting for at least two weeks. In addition at least four of the following symptoms need to be present: significant changes in weight or appetite, changes in sleep patterns, psychomotor agitation or retardation, fatigue or loss of energy, feelings of worthlessness or inappropriate guilt, diminished ability to think or concentrate, and recurrent thoughts of death or suicide. These symptoms must cause clinically significant impairment or distress in the individual's life functioning.

The prevalence of MD in patients with CVD has been reported to be from 16% to 23%^{2,3,4,5}. Even with the use of a stringent diagnostic tool to assess depression, it was found that 18% of patients with CAD were also diagnosed as having a major depressive episode⁶.

It has been suggested that MD after the identification of CVD would be a natural, predictable reaction to this diagnosis although it is clear that MD is an independent risk factor for major cardiac events in patients with coronary artery disease (CAD). Carney et al (1988)² followed 52 patients with CAD, which included 9 patients who met the criteria for MD, according to a structured interview. After a 12-month follow-up period, the incidences of major cardiac endpoints (myocardial infarction, angioplasty, coronary bypass surgery and death) were assessed. Results show that MD was the best single predictor of these endpoints and was independent of severity of CAD and smoking. Interestingly, the prevalence of MD in the general population in the area where the study was conducted was 2.6%, while the prevalence of MD in the patients included in the study was 17%. One limitation of this study was the relatively short follow-up period.

Another study by Barefoot et al (1996)⁷ followed 1,031 patients with CAD for up to 19.4 years. They concluded that MD at time of hospitalization was associated with poorer prognosis in patients with CAD, and this outcome was not related to baseline clinical and anatomic predictors of prognosis, not dependant on initial severity of illness, and not limited to those with the highest probability of recurrent CV events. This study points to the fact that the effects MD may not only be episodic, but may exert influence on the patient over extended periods of time.

There have been numerous studies assessing initially CVD free patients, with and without MD. Ford et al (1998)⁸ assessed 1190 men after graduation from medical school that were initially free of both CVD and MD. The median follow-up time was 37 years. Of the original sample, 132 men reported a subsequent depressive episode. Before correction for conventional risk factors, there was an almost two-fold increase in CHD for the men who reported a depressive episode and even when corrected for conventional risk factors, there remained a clinically significant risk for CHD associated with MD.

Recent studies have examined the relationship between the severity of depression and cardiovascular outcome. Lespérance et al (2003)⁹ followed up 896 post-myocardial infarction (MI) patients after admission for a 1-year period, then further ascertained their 5-year survival using Medicare data. Results have shown that there is an association between the level of depression symptoms at admission for MI and long-term cardiac mortality. Even mild levels of depression were associated with and increased in cardiovascular mortality.

Despite the conclusive evidence that MD and CVD are linked, the mechanism for this association has yet to be established. In this thesis, we propose that nitric oxide (NO) may be a mechanism linking MD and CVD and that antidepressant treatment may affect NO production.

Nitric oxide (NO) consists of one atom of oxygen and one atom of nitrogen. The interest in NO stems from its many and varied physiological actions, from neuromessenger to vasodilator to cytotoxic agent. The biological effect of NO was first discovered by Furchgott & Zawadzki in 1980, when they discovered that the endothelium was necessary for vasodilation to occur¹⁰. They named the hypothesized compound from

the endothelium the endothelial derived relaxing factor (EDRF). Because of similarities in the functioning of NO and EDRF, in 1986 both Ignarro and Furchgott hypothesized that EDRF was NO. Direct chemical and pharmacological evidence that EDRF is in fact NO was first discovered in 1987 ¹¹.

The free radical NO is produced by a five electron oxidation of L-arginine to form both NO and citrulline, with hydroxy-arginine formed as the intermediate ¹². For this reaction to occur, 1.5mol of NADPH and 2 mol of oxygen per mol of L-citrulline are required ¹³. NO is thought to be directly synthesized by three isoforms of the NO synthase (NOS) enzyme. The most common nomenclature is neuronal NOS (nNOS, sometime called brain NOS [bNOS]) for the NOS first purified in neurons, endothelial NOS (eNOS, also seen as ecNOS) first purified in endothelial cells and inducible NOS (iNOS) for NOS from macrophages. There is a further categorization of NOS with eNOS and nNOS called constitutive NOS (cNOS) as one category and iNOS as another. In both platelets and the endothelium only eNOS is characterised.

NOS is made up of 2 monomers that function as a dimer, and can be structurally divided into two domains, the C-terminal and the N-terminal ¹⁴. It is a unique enzyme in that while the C-terminal of the NOS enzyme contains familiar sequences, the N-terminal has very little homology to known proteins ¹⁴. The C-terminal, often called the reductase domain, contains sequences for nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN) ¹⁵. These sequences give it a close homology to cytochrome P450 reductase (CPR) ¹⁶, although its reliance on binding of calmodulin for electron transfer is distinctive ¹⁷. Because of the

similarity of the sequences in the reductase domain to existing proteins it has been well characterized ¹⁸.

The N-terminal region, which is a structurally unique protein ¹⁴ differentiates it from cytochrome P 450. It is thought to contain the L-arginine, tetrahydrobiopterin (BH4) and heme binding sites and is called the oxygenase terminal ¹⁵. The calmodulin binding site is thought to be found where the two terminal regions link ¹².

NO produced by the endothelium or by platelets, plays an important role in the cardiovascular system, as it acts as a vasodilator and also inhibits platelet aggregation. In addition, many cardiovascular diseases have been associated with impairment of the NO system. The first of the following studies is designed to investigate NO as a potential mechanism linking MD and CVD through the measurement of NO metabolites (NOx) in plasma and NOS activity in platelets. The second paper will investigate the effects of paroxetine, a selective serotonin reuptake inhibitor, on both plasma NOx levels and platelet NOS activity in both healthy controls and depressed patients to examine the effect of treatment on NO production.

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CHAPTER 2

Decreased Platelet Nitric Oxide Synthase Activity and Plasma Nitric Oxide Metabolites in Major Depressive Disorder

The content of this chapter are represented in a manuscript submitted for publication in Molecular Psychiatry and that has been provisionally accepted. Authors are **Wendy E. Chrapko**, Paul Jurasz, Marek W. Radomski, Nathalie Lara, Stephen Archer and Jean-Michel Le Mellédo. The author of this thesis played a major role in this study, including design and revision of the protocol, research visits, data collection and analysis and the writing of the manuscript

2.1 INTRODUCTION

An independent association has been found between major depression (MD) and coronary heart disease ¹ (CHD). The prevalence of MD in patients suffering from CHD is high. Indeed, among patients with CHD, the one-month prevalence of MD is approximately 15%, 3 fold higher than the one-month prevalence rate of 5% observed in the community ². In patients suffering from CHD, MD has been associated with a 2.5 to 4 fold increase in the occurrence of cardiovascular events ². In addition, patients with MD initially free of CHD display a greater than average increase risk in CHD ³. The biological explanation for the causal association between MD and CHD remains to be identified.

Nitric oxide (NO) is synthesised from L-arginine by a family of isoformic enzymes known as nitric oxide synthase (NOS). Three isoforms, endothelial, neuronal and inducible NOS (eNOS, nNOS and iNOS respectively) have been identified. The eNOS has been found not only in the endothelium, but also in platelets ^{4,5}. Several cofactors, such as tetrahydrobiopterin (BH4), NADPH (nicotinamide adenine dinucleotide phosphate), flavin mononucleotide, flavin adenine dinucleotide, calmodulin and heme moiety are necessary for the synthesis of NO ⁶.

Numerous cardiovascular diseases have been associated with alterations in the NO system, such as hypertension, heart failure and coronary artery disease ⁷. It has also been found that a specific mutation, the Glu298Asp variant of the eNOS gene is a significant contributing factor for coronary artery disease ⁸ and myocardial infarction ⁹. Many conventional risk factors for CHD, such as smoking ¹⁰, hypercholesterolemia ¹¹,

and physical inactivity¹² are associated with decreased plasma NO metabolite (NOx) levels.

Endothelium-derived NO, through its vasodilator properties, participates in the modulation of vascular tone and inhibits a number of proatherogenic processes such as the oxidation of low-density lipoproteins and the proliferation of smooth muscle cells¹³. Both endothelium- and platelet-derived NO also play a major role in platelet regulation. Physiologically, NO inhibits platelet adhesion to the vascular wall¹⁴ and aggregation⁵. In addition, NO disaggregates preformed platelet aggregates¹⁵ and inhibits activated platelet recruitment to the aggregate¹⁶. The measurement of NO release into blood remains difficult because NO is rapidly inactivated by haemoglobin or oxidized to form several nitrogen dioxides. Plasma NOx concentrations are often used as a marker for vascular NO production¹⁷.

Platelet NOS activity can be measured in isolated human platelet suspensions and used as a sensitive index of platelet function^{18,19}. Platelets play a key role in haemostasis, thrombosis, development of atherosclerosis and acute coronary syndromes. They also play a role in both the development and progression of atherosclerotic lesions²⁰ and endothelial dysfunction²¹. In patients with MD, either with or without comorbid ischemic heart disease, increased baseline platelet activity and responsiveness has been reported²². In addition, baseline levels of platelet factor 4 and β -thromboglobulin, indicators of platelet activation, have been shown to be elevated in patients with MD and ischemic heart disease compared to non-depressed patients with ischemic heart disease²³. It has consequently been suggested that an increase in platelet reactivity accounts for the clotting diathesis and susceptibility to ischemic heart disease in MD²⁴.

Patients suffering from MD with no current CHD also display abnormal endothelial function compared to age, gender and risk factor matched controls²⁵. Endothelial dysfunction has been linked to many cardiovascular diseases such as atherosclerosis and this is detectable prior to the development of vascular lesions, even in the first decade of life²⁶. Many of the mechanisms that have been proposed to explain the impaired endothelium-dependant relaxation in atherosclerosis involve decrease in endothelial-derived NO production, i.e., a decrease in eNOS gene expression and a decrease in the necessary cofactors for NO synthesis²⁶.

The mechanisms explaining the association between MD, increased platelet reactivity, and endothelial dysfunction, along with their impact on increased cardiovascular risk, remains to be determined. One potential explanation is that MD leads to a decrease in NO production by platelets and by the endothelium. Therefore, the aim of this paper was to better elucidate the mechanisms that could be implicated in the increased cardiovascular risk associated with MD. For this, we examined NO production, as measured by NOS activity and plasma NOx levels, as well as BH4 levels, in patients with a current diagnosis of MD and in healthy controls (HC). We also hypothesised that decreased NO levels in platelets and plasma in patients with MD will not be related to factors known to reduce NO production such as cholesterol levels, physical activity or smoking.

2.2 METHODS

2.2.1 Subjects and General Procedures

Subjects were 16 HCs (9 males and 7 females, mean age 27.13 standard error of mean (SEM) $\pm$ 2.53) and 15 patients diagnosed with current MD (10 males and 5

females, mean age 33.07 SEM $\pm$ 2.25). Subjects were excluded if they were smokers, had diabetes, past or current CHD, or a family history of early CHD. All subjects were recruited through advertisements and were assessed using the Structured Clinical Interview for DSM-IV Axis I Disorders, to ensure accuracy and standardisation of psychiatric diagnoses²⁷. HCs did not have a diagnosis of a current or past psychiatric Axis I disorder. The Hamilton Depression Rating Scale and Beck Depression Inventory were administered in the screening visit and in the MD patients, the means were 20.40 (SEM $\pm$ 1.15) and 24.80 (SEM $\pm$ 1.00) for the scales respectively, indicating mild to moderate severity of the current depressive episode. In comparison, the HCs had a mean Hamilton Depression Rating Scale score of 1.38 (SEM $\pm$ 0.41) and a mean Beck Depression Inventory score of 1.56 (SEM $\pm$ 0.42). Of the 15 MD patients, 11 subjects had prior episodes of depression (range 1 prior episode to 11 prior episodes). Of those with prior episodes, 7 had received prior pharmacological treatment, 1 had been previously hospitalized for MD and 3 subjects had previously attempted suicide. The inclusion of MD patients of mild severity in the MD group is justified by the fact that mild depression has also been shown to carry increased cardiovascular risk²⁸. Depressed subjects with a co-morbid psychiatric disorder, except those with anxiety disorders, were excluded. The inclusion of patients with MD and anxiety disorders is justified by the high prevalence of this comorbidity²⁹. Of the 15 MD patients, 9 had a comorbid anxiety disorder. Three patients with MD also suffered from Generalised Anxiety Disorder, 2 had Social Phobia, 1 had Obsessive-Compulsive Disorder and 1 had Post Traumatic Stress Disorder. In addition, one patient was diagnosed with both Generalised Anxiety Disorder and a Specific Phobia while another was diagnosed with Generalised Anxiety Disorder, a

Specific Phobia and Social Phobia. None of the research subjects have taken any antidepressant or psychotropic medications in at least the last year (range 1 to 15 years).

All subjects gave written consent after a full written and verbal explanation of the study. Ethical approval was obtained from the Health Research Ethics Board at the University of Alberta prior to the commencement of the study. Data collection for both MD patients and HCs were undertaken concurrently over the same 18-month period. Blood samples for all subjects were taken at approximately the same time during the day (within 2 hours). Subjects came in for the blood collection after following a low nitrate diet for 3 days, to control for the confound of dietary nitrate³⁰, and fasting for a minimum of 12 hours for cholesterol measurements. Subjects were also asked to refrain from taking any medication for 3 days prior to sampling as various commonly taken medications, such as ibuprofen, have been shown to alter NO production³¹. All blood collection for female patients was scheduled prior to midcycle, as NOx levels peak during this time of the cycle³². Physical activity levels were measured using the 7-day Recall Interview³³.

2.2.2 Platelet Isolation

Platelet isolation was performed as described by Radomski et al. 1983¹⁸. Briefly, peripheral venous blood (36mL) was added to 4ml of 3.15% (w/v) tri-sodium citrate. Prostacyclin (PGI₂, 0.3 μ M) was added to citrated blood and the samples were centrifuged at 400 x g for 20 minutes. The platelet rich plasma was then collected and further centrifuged at 800 x g in the presence of 1 μ M PGI₂ to obtain the platelet pellet. Platelets were then gently resuspended in Tyrode's buffer to remove contaminating red blood cells, and platelet suspension was centrifuged at 800 x g in the presence of 1 μ M

PGI₂. The platelet pellet was washed three times with PGI₂-free Tyrode's solution (1 mL) and then frozen at -80°C until assayed for NOS activity.

2.2.3 Determination of Platelet Nitric Oxide Synthase Activity

NOS activity was determined as described before by Radomski et al 1993¹⁹. Briefly, washed platelet pellets were homogenized on ice using a Vibra Sonic sonicator (Sonics & Materials Inc. Danbury, CT). The NOS activity was determined by measuring the conversion of L-[¹⁴C]arginine to L-[¹⁴C]citrulline. The dependence of L-arginine conversion on Ca²⁺-dependent (constitutive) NOS was confirmed using EGTA and N(G)-mono-methyl-L-arginine (L-NMMA), an inhibitor of this enzyme.

The sensitivity of this assay has been shown to be just under 1 pmol/min/mg protein for the detection of L-[¹⁴C]arginine. The specificity of this analysis is guaranteed through the use of EGTA, which allows us to see if inducible NOS activity is present and through the use of L-NMMA which acts as a control. In addition, any non-specific conversion of L-[¹⁴C]arginine is subtracted. Assay was conducted blind to clinical information and with equal distribution between both MD patients and HCs in each run of the assay to control for any potential inter-assay variability.

2.2.4 Determination of Nitric Oxide Metabolite levels

Peripheral venous blood samples were collected immediately after collection of samples for NOS analysis into a 5ml vacutainer tube containing EDTA centrifuged at 3000 RPM at 4°C for 10 minutes. Plasma samples were stored at -20°C until the time of analysis.

All samples were analysed using the chemiluminescence method³⁴. Briefly, NOx in small amounts of plasma can be converted into NO using a strong reducing

environment, namely vanadium (III) chloride in 1N HCl at 93.3°C, and can then be detected by an NO Analyser (NO Analyser 280; Sievers Instruments, Boulder CO). The sensitivity of this analyser has been ~ 1 picomole of NO injected into the purge vessel. This analysis will convert almost all nitrate and nitrite to NO and thus the importance of a nitrate free diet. Plasma samples were diluted with an equal volume of double deionised NO free water to decrease sample viscosity. An antifoaming agent (Dow Corning, Midland, MI) was added to the reagent to avoid foaming caused by plasma proteins. NOx levels are reported in $\mu\text{mol/L}$. For this analysis, assays were not conducted blind to clinical information but this is unlikely to have affected the final results since the areas for the NOx curves only were collected in the lab while the calculations of NOx levels against standards were conducted at a later date. To avoid the potential confound of inter-assay variability, equal distribution of samples from both MD patients and HCs were used in each run of the assay.

2.2.5 Determination of Tetrahydrobiopterin Levels

For the analysis of BH₄, serum samples were acidified with 0.2N HCl and the proteins were precipitated with 30% trichloroacetic acid. The resulting supernatant was treated with manganese dioxide to oxidise the BH₄ to biopterins. The resultant mixture was centrifuged and the supernatant was filtered through 0.22 μm filters (Chromspec) and transferred into autosampler vials. The extracted samples were analysed using Hewlett Packard HPLC 1100. A reverse phase sphereclone column, 5 μm ODS (Phenomenex) was used. The mobile phase was 5% methanol and the fluorescence detector was used at 350nm excitation and 410nm emission. The retention time for the biopterin was 6.4 min.

2.2.6 Statistical Analysis

The Mann Whitney U test was used for the analysis of the differences between MD subjects and HCs. Values are expressed as mean $\pm$ standard error of mean (SEM). A p value of less than 0.05 was considered significant. All statistical analyses were conducted using SPSS statistical software for Windows (SPSS Inc. Chicago, IL).

2.3 RESULTS

Both platelet NOS activity and NOx plasma levels were lower in MD compared to HCs. Mean plasma NOx levels were 13.68 $\mu\text{mol/L}$ (± 2.85) for subjects with MD compared to 32.37 $\mu\text{mol/L}$ (± 2.36) for HCs ($p < 0.01$; see Figure 2-1). Mean platelet NOS activity levels were 0.89 pmol/min/mg protein (± 0.41) for those with MD compared to 17.05 pmol/min/mg protein (± 2.93) for HCs ($p < 0.01$; see Figure 2-2). No group differences were found for the potential confounding variables measured including age, body mass index, cholesterol levels, and blood pressure (see Table 2-1). The differences in NOS activity and NOx levels remain statistically significant when MD patients with or without co-morbid anxiety disorders were compared separately to HCs. No statistically significant differences were found between MD subjects with co-morbid anxiety disorder(s) and MD alone in NOS activity (0.90 ± 0.60 pmol/min/mg protein vs. 0.88 ± 0.53 pmol/min/mg protein respectively, $p = 0.98$) or plasma NOx levels (15.26 ± 5.28 vs. 11.32 ± 3.36 respectively, $p = 0.52$). We did not find a correlation, using Pearson's correlation, between severity of MD and platelet NOS activity or plasma NOx levels, whether measured by the Hamilton Depression Rating Scale ($p = 0.39$ and $p = 0.52$ respectively) or with the Beck Depression Inventory ($p = 0.60$ and $p = 0.50$

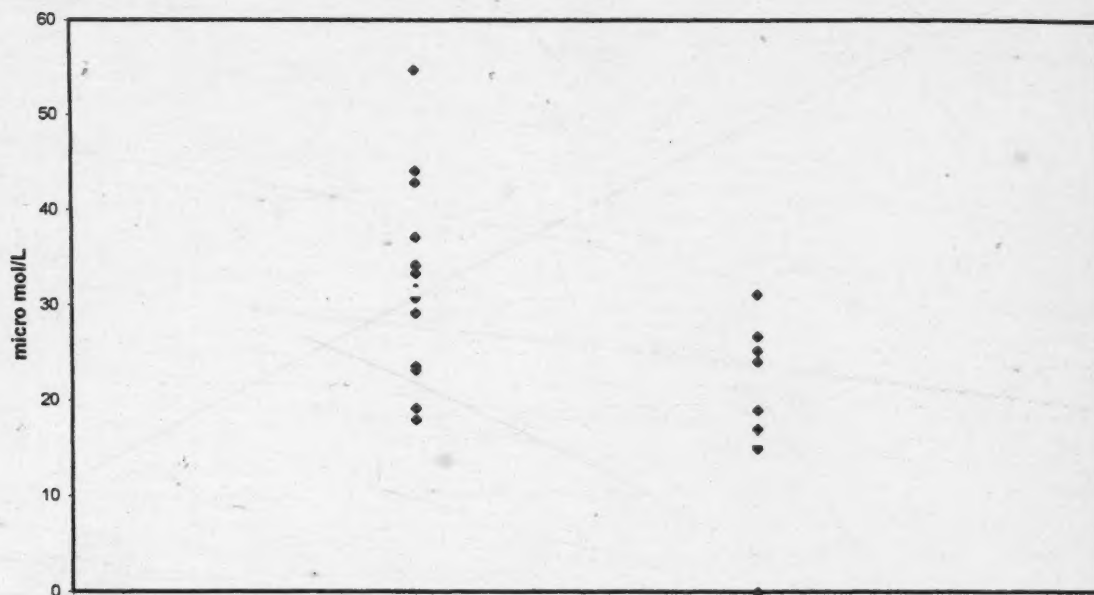


Figure 2-1. Scatter graph of individual variables for plasma nitric oxide metabolite (NOx) levels in patients with major depression (MD) versus healthy controls (HCs).

Means and standard error of means for MD patients = $13.68 \mu\text{mol/L} (\pm 2.85)$, Mean and standard error of mean for HCs = $32.37 \mu\text{mol/L} (\pm 2.36)$, Median for MD = $15.82 \mu\text{mol/L}$ (10 MD patients had undetectable concentrations), Median for HCs = $31.39 \mu\text{mol/L}$.

Medians are indicated by horizontal bars.

Table 2-1. Demographic and Laboratory Values in Study Subjects

Patient Characteristic	MD	HC	p value
Physical Activity (kcal/kg/day)	34.84 ($\pm$ 0.84)	35.51 ($\pm$ 0.49)	0.49
LDL-Cholesterol (mg/L)	119.36 ($\pm$ 9.40)	99.56 ($\pm$ 9.44)	0.15
HDL - Cholesterol (mg/L)	44.38 ($\pm$ 2.96)	48.84 ($\pm$ 2.43)	0.25
Total Cholesterol (mg/L)	188.67 ($\pm$ 10.98)	169.67 ($\pm$ 8.51)	0.18
Triglycerides (mg/L)	54.89 ($\pm$ 6.94)	54.01 ($\pm$ 14.04)	0.96
BH4 (nM)	27.31 ($\pm$ 3.64)	27.04 ($\pm$ 2.47)	0.95
Folate (nmol/L)	867.62 ($\pm$ 86.41)	827.00 ($\pm$ 51.10)	0.68
Age (years)	33.07 ($\pm$ 2.25)	27.13 ($\pm$ 2.35)	0.08
BMI (kg/m ²)	29.4 ($\pm$ 2.29)	25.7 ($\pm$ 1.86)	0.19
SBP (mm Hg)	119.9 ($\pm$ 3.32)	119.2 ($\pm$ 3.27)	0.89
DBP (mm Hg)	81.8 ($\pm$ 1.71)	78.6 ($\pm$ 3.16)	0.39
Heart rate (beats/minute)	68.4 ($\pm$ 1.55)	68.5 ($\pm$ 2.72)	0.95
BMI = Body Mass Index; SBP = systolic blood pressure; DBP = diastolic blood pressure; HDL = high-density lipoprotein; LDL = Low-density lipoprotein; BH4 = tetrahydrobiopterin			

respectively). In addition, we did not find a correlation between plasma NOx levels and platelet NOS activity using Pearson's correlation coefficient in the MD patients ($p = 0.62$) or in the HCs ($p = 0.27$). There were no statistically different gender differences in either NOS activity or NOx levels ($p = 0.34$ and $p = 0.07$ respectively).

2.4 DISCUSSION

We have found that both platelet NOS activity and plasma NOx levels are decreased in MD patients. This study is the first to examine both plasma NOx and platelet NOS activity in MD. Suzuki et al (2001)³⁵ examined plasma nitrate levels in subjects with MD using both patients with an anxiety disorder and HCs as a comparison and found elevated nitrate levels in patients with MD using high-performance liquid chromatography. However, in that study, the patients with MD were taking a variety of psychotropics, in particular antidepressant medications (imipramine, amitriptyline and mianserin) so that any comparison between patients with MD while on medication and HCs who are not taking the same medication is questionable. Indeed, we have recently shown that the antidepressant paroxetine, which share similar mechanisms of action with some of the antidepressants used in the study by Suzuki et al, increase plasma NOx levels in HCs³⁶. Similar remarks could be made about an interesting study conducted by Savas et al (2002)³⁷ which investigated a potential role of NO in the pathophysiology of bipolar disorder (type I, manic episodes).

To ensure that the changes in NO levels were associated with MD, but not with confounding factors, smokers were excluded from this study and conventional risk factors known to affect NO production were controlled for. For example, since hypercholesterolemia has been shown to induce a decrease in NO production³⁸,

cholesterol plasma levels were measured. The lack of difference in cholesterol levels between MD patients and HCs indicates that the decreased NO production in MD patients is independent of cholesterol levels.

In addition, we did not find differences in NOx plasma levels and platelet NOS activity between the MD patients with and without comorbid anxiety disorders and both these subgroups, when compared to HCs, displayed decreased plasma NOx and platelet NOS activity. This suggests a lack of a major confounding effect of comorbid anxiety disorder on our results. However, due to the limited representation of each anxiety disorder in our sample, it cannot be excluded that certain anxiety disorders are associated with specific dysregulation of endothelial NO production and platelet NOS activity. We have in fact undertaken investigations of dysregulation of peripheral measurements of NO in various anxiety disorders in patients not displaying comorbid MD.

Contrary to previous reports of lower BH4 plasma levels in MD³⁹, we did not observe differences between the levels of this NOS co-factor in MD patients and HCs. Therefore, the mechanism(s) responsible for the decreased NO production in MD remains to be determined.

The values for plasma NOx and platelet NOS activity in our HCs are well within the normal range, which supports the validity of our data. In moderate chronic smokers, a decrease in plasma NOx of 22% was reported compared to HCs, while in heavy smokers this relative decrease was 36%¹⁰. In patients with hypercholesterolemia, a 33% decrease in mean plasma NOx levels were observed, with individual NOx levels negatively correlated with LDL cholesterol levels⁴⁰. In comparison, we have found a 92% decrease in plasma NOx levels in MD patients compared to HCs suggesting that this reduction is

of clinical significance. Indeed, the dramatic decrease in levels of plasma NOx and platelet NOS activity in patients with MD may be a contributing factor to the increased cardiovascular risk associated with MD.

The decrease in plasma NOx that we observed in MD patients suggests that the production of NO by the endothelium is decreased in MD patients. NO is an essential contributor to endothelium function. Endothelial dysfunction is associated with cardiovascular diseases such as atherosclerosis as well as with conventional cardiovascular risk factors such as smoking, hypercholesterolemia and inactivity. Drugs that improve outcome in atherosclerotic diseases (e.g. statins, angiotensin-converting enzyme inhibitors) also improve endothelial function. Furthermore endothelium dysfunction precedes overt vascular disease by years ⁴¹. The decrease in endothelial NO production in MD patients might therefore be the cause of the endothelium dysfunction found in MD patients and therefore be responsible for the future development of overt symptoms of vascular disease.

Platelet activation is critical in the development of atherosclerosis and acute coronary syndromes. Both endothelium and platelet derived NO contribute to preventing platelet adhesion to the vascular wall and platelet aggregation therefore playing a key role in the prevention of thrombus formation which is a major trigger of coronary accidents. The decrease in endothelial NO production and platelet NOS activity may contribute to the observed increased-platelet reactivity found in patients with MD ⁴² and therefore to the increase risk for CHD and acute coronary syndromes described in patients with MD.

In this study we have not attempted to measure other indices of platelet and endothelial functions such as adhesion, aggregation and vascular reactivity. Therefore,

future investigations are needed to replicate these results in a larger sample and attempt to link the decrease in plasma NOx and platelet eNOS activity in MD patients to platelet and endothelial dysfunctions and ideally to cardiovascular outcomes.

It is uncertain whether the NO dysregulations associated with MD are state or trait dependent. This indirectly relates to the crucial question of whether or not the cardiovascular risk of MD patients can be modified by antidepressant treatment ⁴³.

Valuable information regarding the cardiovascular risk associated with MD could be gathered by assessing the effect of successful antidepressant treatment on NO production in MD patients. Our preliminary data showing that treatment with paroxetine, a selective serotonin reuptake inhibitor, increases NO production ³⁶ are interesting in this regard.

In conclusion, our study proposes novel NO-related mechanisms that link MD with CHD.

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CHAPTER 3

Paroxetine-induced Changes in Levels of Plasma Nitric Oxide Metabolites and Platelet Nitric Oxide Synthase Activity in Subjects with Major Depressive Disorder Compared to Healthy Controls

The content of this chapter are represented in a manuscript to be submitted for publication in American Journal of Psychiatry. Authors are **Wendy E. Chrapko**, Paul Jurasz, Marek W. Radomski, Stephen Archer, Stephen C. Newman, Glen Baker and Jean-Michel Le Mellédo. The author of this thesis played a major role in this study, including design and revision of the protocol, research visits, data collection and analysis and the writing of the manuscript

3.1 INTRODUCTION

The link between major depressive disorder (MD) and cardiovascular disease (CVD) has been well established. In prospective studies using initially healthy subjects, MD has been found to be an independent risk factor for both first myocardial infarction and cardiovascular mortality, with a range of 1.5 to 2 for adjusted relative risk¹. In addition, patients suffering from CVD who are also diagnosed with MD have a 2.5 to 4-fold increase in the occurrence of cardiovascular events². Although still unclear, both endothelial dysfunction and platelet hyperactivity have been suggested as two of the main mechanistic explanations for the link between MD and CVD³.

Recent studies have alluded to a potential role of nitric oxide (NO) in the relationship between major depression and cardiovascular risk as well as an effect of antidepressants on NO production^{4,5,6}. NO produced from either the endothelium or from platelets plays a major role in cardiovascular regulation and cardiovascular pathology. Indeed, numerous cardiovascular diseases, such as hypertension, heart failure and coronary artery disease, have been associated with alterations in the NO system⁷. NO is not only responsible for vasodilatation of the endothelium, but also inhibits smooth muscle cell proliferation and migration, oxidation of low-density lipoproteins and platelet aggregation and adhesion. It also disaggregates preformed platelet aggregates⁸ and inhibits activated platelet recruitment to the aggregate⁹. NO is synthesised from L-arginine by a family of isoformic enzymes known as nitric oxide synthase (NOS). Three isoforms, namely endothelial, neuronal and inducible NOS (eNOS, nNOS and iNOS respectively), have been identified. The eNOS isoform has been found not only in the endothelium, but also in platelets^{10,11}. Endothelial dysfunction precedes overt vascular

disease, even in the first decade of life¹². Levels of plasma nitric oxide metabolites (NOx) have been used as markers for endothelial NO production¹³. It has also been suggested that impaired platelet production of NO can predict the presence of acute coronary syndromes¹⁴. In isolated human platelet suspensions, platelet NOS activity can be measured^{15, 16}.

In recent years, selective serotonin reuptake inhibitors (SSRIs) have become the most commonly prescribed treatment for MD, specifically in a population with comorbid depression and cardiovascular disease¹⁷. Finkel et al (1996)¹⁸ found that in subjects with ischemic heart disease being treated for MD, the SSRI paroxetine reduced levels of NO, suggesting a potentially deleterious effect of paroxetine on the cardiovascular system since decreased endothelium production of NO is usually associated with increased cardiovascular risk¹⁹. However, numerous studies have found that SSRIs, including paroxetine, are a safer treatment option in a population with CVD and MD in comparison to tricyclic antidepressants^{20, 21, 22}.

Intrigued by the finding of Finkel et al (1996)¹⁸, our research team investigated the effects of paroxetine on plasma NOx levels in a group of male healthy controls (HCs)⁶. In that study, administration of paroxetine resulted in an increase in plasma NOx levels after 8 weeks of administration. Once the drug was discontinued, levels of NOx returned to normal levels. This is particularly interesting as we have also shown that plasma NOx levels and platelet NOS activity are extremely low in unmedicated MD patients without conventional cardiovascular risk factors²³. The correction of the decreased NO production by paroxetine treatment could have a potentially cardioprotective effect in patients with MD. Musselman et al (2000)²⁴ have shown that 6 weeks of paroxetine

normalised increased platelet reactivity in MD patients. This effect could be mediated by a paroxetine-induced increase in platelet NOS activity since the L-arginine-NO pathway is a negative feedback mechanism to suppress excessive platelet activation and aggregation²⁵.

The purpose of the study reported here was to explore the effects of paroxetine on the endothelium and platelets in both male and female patients suffering from MD or HCs. We hypothesized that both plasma NOx levels and platelet NOS activity will increase after administration of paroxetine in both subject groups.

3.2 METHODS

3.2.1 Subjects, Design and General Procedures

Subjects were 12 HCs (7 males and 5 females, mean age 26.75 ± 2.78) and 17 patients diagnosed with current MD (11 males and 6 females, mean age 34.50 ± 2.08).

All subjects were assessed using the Structured Clinical Interview for DSM-IV Axis I Disorders. No HCs were diagnosed with either current or past psychiatric Axis I disorder. MD patients with a diagnosis of a comorbid anxiety disorder, but no other Axis I disorder such as bipolar disorder or schizophrenia, were also included because of the frequency of such comorbidity²⁶. The diagnosis of MD was the primary diagnosis. The mean score on the Hamilton Depression Rating Scale²⁷ for the MD patients was 20.71 ± 4.25 and on the Beck Depression Inventory²⁸ the mean score was 25.18 ± 4.08 , indicating that most patients were in the moderate range of severity. All MD patients were free of psychotropic medications for at least one year. Classical cardiovascular risk factors (blood pressure, cholesterol levels, and physical activity) were measured at baseline. Physical activity levels were measured using the 7-day Recall Interview²⁹.

One male MD patient was a smoker and consistently smoked approximately half a pack of cigarettes per day. Since acute smoking directly affects NO levels³⁰ he was asked to refrain from smoking prior to any visit where blood samples were taken. It was also established that subjects did not have current or past CVD or a family history of early cerebro-cardiovascular illness.

In total, 40 subjects (16 HCs and 24 with MD) began the study. Of the HCs, one (male) withdrew due to time constraints and 3 HCs (2 males and 1 female) withdrew due to side effects, anorgasmia for both males and nausea for the female. Of the MD subjects, 5 subjects with MD (1 female and 4 males) were withdrawn from the study due to non-compliance (for example, missing appointments or irregular use of medications). One female MD patient withdrew after the screening due to extremely high levels of anxiety. Another female MD patient was withdrawn due to her very irregular menstrual cycles, which made the accurate and regular scheduling of her appointments according to her menstrual cycle (see below) impossible. All subjects gave written consent after receiving a full written and verbal explanation of the study. Ethics approval was obtained from the Health Research Ethics Board at the University of Alberta prior to the commencement of the study.

Each subject came in for a total of 7 visits, which correspond to a baseline visit, visits after 1, 2, 4, 6 and 8 weeks of treatment, and then 1 visit after discontinuation of the medication. As described below, only at certain visits were blood samples taken. During all visits, side effects of the paroxetine and severity of MD symptoms were assessed. Experimental design in the collection of the NOx blood samples varied slightly between male and female subjects, due to the need to control for menstrual cycle, as different

phases of the menstrual cycle can effect NO production³¹. For both MD and HC males, venous blood samples were taken at baseline, after 2 weeks, 6 and 8 weeks of treatment and between 8 and 12 days after discontinuation of paroxetine. For both MD and HC females, venous blood samples were taken at baseline, after 8 weeks of treatment and again after discontinuation for one full menstrual cycle with all visits scheduled prior to midcycle, as NOx levels peak during this time of the menstrual cycle³¹. Due to the large amount of blood required (approximately 40 mL) and the time-intensive nature of the NOS assay, for both males and females, venous blood samples for the analysis of platelet activity were collected only at baseline and after 8 weeks of treatment. These samples were collected during the same visits as the plasma samples for NOx analysis.

Prior to every visit with blood collection, subjects fasted for a minimum of 12 hours and followed a low nitrate diet for 3 days, to control for the confounding effects of dietary nitrate³². Subjects were also asked to refrain from taking any medication for 3 days prior to sampling as various commonly taken medications, such as ibuprofen, have been shown to alter NO production³³.

During the baseline visit paroxetine was prescribed, with treatment to start the following day. Paroxetine was administered initially at a dose of 10mg/day for 3 days then increased to 20mg/day for the remainder of the study. Side effects were frequently reported and were those generally associated with the administration of SSRIs, such as nausea, changes in bowel movements, and sexual dysfunction. However, these were tolerated well by both the MD patients and HCs who completed the study. Discontinuation of the medication was gradual to prevent withdrawal effect, and tapered to 10mg/day for 5 days then stopped.

3.2.2 Platelet Isolation

The method for platelet isolation was performed as described previously in Radomski et al. 1983¹⁵. Briefly, 40mL of peripheral venous blood from the antecubital vein was added to 4ml of 3.15% (w/v) tri-sodium citrate. Prostacyclin (PGI₂, 0.3 mM) was added to the citrated blood, which was then centrifuged at 400 x g for 20 minutes. To obtain the platelet pellet, the platelet-rich plasma (upper layer) was then collected and further centrifuged at 800 x g in the presence of 1 mM PGI₂. Platelets were then gently resuspended in Tyrode's buffer to remove contaminating red blood cells, and the platelet suspension was centrifuged at 800 x g in the presence of 1 mM PGI₂. The platelet pellet was then washed three times with PGI₂-free Tyrode's solution (1 mL) and frozen at -80°C until assayed for NOS activity.

3.2.3 Determination of Platelet Nitric Oxide Synthase Activity

NOS activity was measured using the method of Radomski et al (1993)¹⁶. Briefly, the platelet pellets were homogenized on ice using a Vibra Sonic sonicator (Sonics & Materials Inc. Danbury, CT). The conversion of L-[¹⁴C]arginine to L-[¹⁴C]citrulline was used as a measure of NOS activity. The dependence of L-arginine conversion on Ca²⁺-dependent NOS was confirmed using EGTA and N(G)-monomethyl-L-arginine (L-NMMA), an inhibitor of this enzyme. Platelet NOS activity is reported in pmol/min/mg protein.

3.2.4 Determination of Nitric Oxide Metabolite levels

Peripheral venous blood samples were collected into a 5ml vacutainer tube containing EDTA. They were then stored on ice until they could be centrifuged (at 1000 x g at 4°C for 10 minutes) to isolate the plasma.

The chemiluminescence method was used to analyse NOx levels in the plasma³⁴. Briefly, NOx in small amounts of plasma can be converted back into NO using vanadium (III) chloride in 1N HCl at 93.3°C and can then be detected by an NO Analyser (NO Analyser 280; Sievers Instruments, Boulder CO). To decrease plasma viscosity, samples were diluted with an equal volume of double deionised NO⁻ free water. To avoid foaming, an antifoaming agent (Dow Corning, Midland, MI) was added to the reagent. Plasma NOx levels are reported in $\mu\text{mol/L}$.

3.2.5 Determination of Paroxetine Levels

To monitor compliance, a blood sample was taken at 8 weeks into treatment to measure plasma paroxetine levels in both males and females. Subjects had taken the paroxetine at between 1.5 and 3 hours prior to collection of the blood sample and thus were in the absorption phase at the time of sampling. Plasma paroxetine levels were analysed using the gas chromatographic procedure of Lai et al. (2000)³⁵. Briefly, this involves basification of the samples, extraction with ethyl acetate, derivatization with heptafluorobutyric anhydride and analysis on a gas chromatograph (Hewlett Packard 5890) equipped with a capillary column and an electron-capture detector.

3.2.6 Statistical Analysis

An independent samples t-test was used to determine if there were significant differences between baseline plasma NOx levels and NOS activity in the HCs and subjects with MD. Since either heavy or light smoking influences NO production³⁶ for this analysis the male MD patient that smoked was excluded from this analysis. In addition, NOx levels were missing from one MD subject and NOS levels were missing from another MD subject due to difficulties in drawing the blood samples.

For the statistical analysis of differences in plasma NOx levels, a linear mixed effects model ³⁷ was used, that is, one in which both random and fixed effects appear linearly. This model has advantages over classical linear regression and repeated measures analysis of variance: intra- as well as inter-individual variation can be modelled, correlated observations can be analyzed, and missing data are permitted. Both a random intercept and a random slope model were fit to the data. The statistical analysis was performed using the SAS procedure PROC NL MIXED (SAS Institute Inc., 1999). To confirm these results using a more traditional approach, a weighted linear regression was performed, with adjustments made for correlated observations. *Post hoc* analyses, in the form of paired t-tests, were conducted to determine when changes in NOx levels became significantly different from baseline and to compare baseline blood samples to those taken after discontinuation of paroxetine.

For the statistical analysis of platelet NOS activity, a 2-way ANOVA was used to measure the differences between baseline visits and after 8 weeks of treatment for all subjects with data for both visits, namely 16 MD subjects and 11 HCs. A *post hoc* within-subjects t-test was performed to examine the significance of changes between baseline and end of treatment measurements. Finally, an independent samples t-test was performed to determine if there was a statistically significant difference between the MD patients and HCs for the samples drawn after 8 weeks of treatment.

Because of a very low plasma level of paroxetine in one subject taken during the visit 8 weeks in to treatment, this subject was excluded from portions of the analysis since his compliance to treatment was in question. The subject was included in the baseline samples analysis (prior to treatment) and the mixed effects model for the

analysis of plasma NOx levels. Results remained statistically significant whether this subject was included or excluded from the mixed effects models.

In the initial weighted linear regression and mixed effects analyses, the one male MD patient that was also a smoker was included. To ensure that this subject was not affecting the results, he was then excluded from the analysis with no discernable effect.

An independent samples t-test was used to determine if there were any significant differences between the HCs and MD patients with regard to a variety of factors that have been known to affect NO production.

Unless otherwise indicated, statistical analyses were conducted using SPSS statistical software for windows (SPSS Inc. Chicago IL). Values are expressed as means $\pm$ standard deviations. A p value of less than 0.05 was considered significant in all analyses.

3.3 RESULTS

Baseline plasma NOx levels were lower in MD patients compared to HCs (5.79 ± 6.33 $\mu\text{mol/L}$ and 22.21 ± 13.02 $\mu\text{mol/L}$ respectively, $t=4.30$, $df=25$, $p<0.001$) and baseline platelet NOS activity was also decreased in MD patients compared to HCs (1.00 ± 1.56 $\text{pmol/min/mg protein}$ versus 15.70 ± 9.55 $\text{pmol/min/mg protein}$ respectively, $t=5.89$, $df=25$, $p<0.001$).

Figure 3-1 shows plasma NOx levels in MD patients and HC subjects. At each observation time, plasma NOx levels are lower in MD patients and for both groups those levels demonstrate a parabolic trajectory over time. Mixed effects models were fit with NOx level as the dependent variable and with fixed effects for study group (modelled as dummy variable: 1=MD, 0=HC), time-squared, age and gender, and with random effects

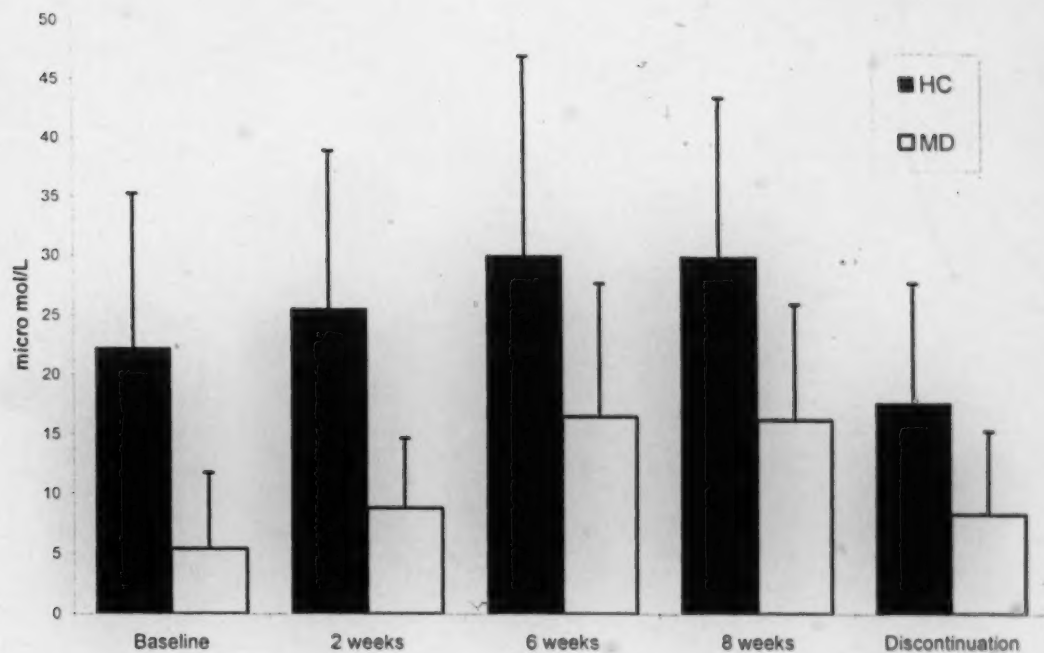


Figure 3-1. Plasma nitric oxide metabolite levels in major depressive patients compared to healthy controls before and during treatment and after discontinuation of paroxetine

Legend for Figure 3-1. Values are means $\pm$ standard deviation.

for intercept and slope. The final model was a random intercept model with terms for study group and time-squared (Table 3-1); an interaction term for study group and time was not statistically significant. The presence of a random coefficient for intercept indicates that study subjects varied in the baseline value of NOx. The weighted linear regression analysis produced a final model with the same (fixed) effects, having almost identical parameter estimates, but with somewhat smaller p-values.

Paroxetine treatment progressively increased plasma NOx levels in both HCs and MD patients (Figure 3-1). Since there was no interaction between study group and time on plasma NOx samples, evidenced by the lack of significance of an interactive term in the random intercept model, the HCs and MD patients were grouped together for *post hoc* the analysis aimed at determining the chronology of the increases from baseline in plasma NOx levels induced by paroxetine. By the second week of treatment, there was a statistically significant difference from baseline measurements ($t=-2.49$, $df=16$, $p=0.02$); this effect was still evident at 6 weeks and at 8 weeks ($t=-3.42$, $df=17$, $p<0.01$ and $t=-5.91$, $df=27$, $p<0.01$ respectively). There was no statistically significant difference between measures at baseline and after discontinuation of the paroxetine ($t=0.30$, $df=24$, $p=0.77$) suggesting that after paroxetine discontinuation plasma NOx levels return to pre-treatment levels. In both the MD patients and HCs there appears to be a plateauing of the plasma NOx levels in that there is no longer a statistically significant difference in the means between the 6 and 8 week visits ($t=-0.81$, $df=17$, $p=0.43$).

Based on the analysis of platelet NOS activity in all patients, a main effect for time and a time by study group effect was found ($F=23.90$, $df=1$, $p<0.001$

Parameter	Estimate	Standard Error	t Value	p-value
<u>Fixed effects</u>				
Intercept	30.72	2.70	11.37	< 0.0001
MD	-13.69	3.27	-4.19	0.0003
Time squared*	-0.41	0.07	-6.06	< 0.001
<u>Random effects</u>				
Intercept	61.55	19.71	3.12	.0043
NOx	45.50	6.81	6.68	<.0001

Table 3-1. Analysis using the linear mixed effects model for NOx levels in MD compared to HC.

Legend for Table 3-1: * time variable centred at 5.0

and $F=34.84$, $df=1$, $p<0.001$ respectively; see Figure 3-2). *Post hoc* analysis indicated that there was a significant decrease in platelet NOS activity in the HCs, from 16.41 ± 9.68 pmol/min/mg protein at baseline to 1.44 ± 1.71 pmol/min/mg protein after 8 weeks of paroxetine treatment ($t=4.99$, $df=10$, $p = 0.001$). However, there was no significant change in platelet NOS activity in the MD group (0.94 ± 1.53 pmol/min/mg protein at baseline versus 2.34 ± 3.75 pmol/min/mg protein after 8 weeks of paroxetine treatment, $t=-1.34$, $df=15$, $p = 0.200$). There was also no statistically significant difference in platelet NOS activity between the MD patients and HCs after 8 weeks of treatment ($t=0.75$, $df=25$, $p = 0.463$).

There were no apparent differences in plasma NOx levels and platelet NOS activity between the responders to paroxetine treatment ($n=11$) and non-responders ($n=6$) as defined by at least a 50% improvement on the Hamilton Depression Rating Scale score in MD patients ($t=0.128$, $df=14$, $p=.90$ and $t=0.53$, $df=14$, $p=0.61$ respectively).

Using Pearson's correlation coefficient, we did not find a correlation between platelet NOS activity and plasma NOx levels in HCs after 8 weeks of treatment (correlation coefficient=-0.22, $p=0.51$).

No significant differences were found between the HCs and MD patients with regard to a variety of factors that have been known to affect NO production and thus may have confounded the results (see Table 3-2).

3.4 DISCUSSION

The results of the current study replicate the findings of Chrapko et al. (submitted)²³ which found lower plasma NOx levels and platelet NOS activity in both male and female patients with MD compared to male and female HCs. It should be noted that there

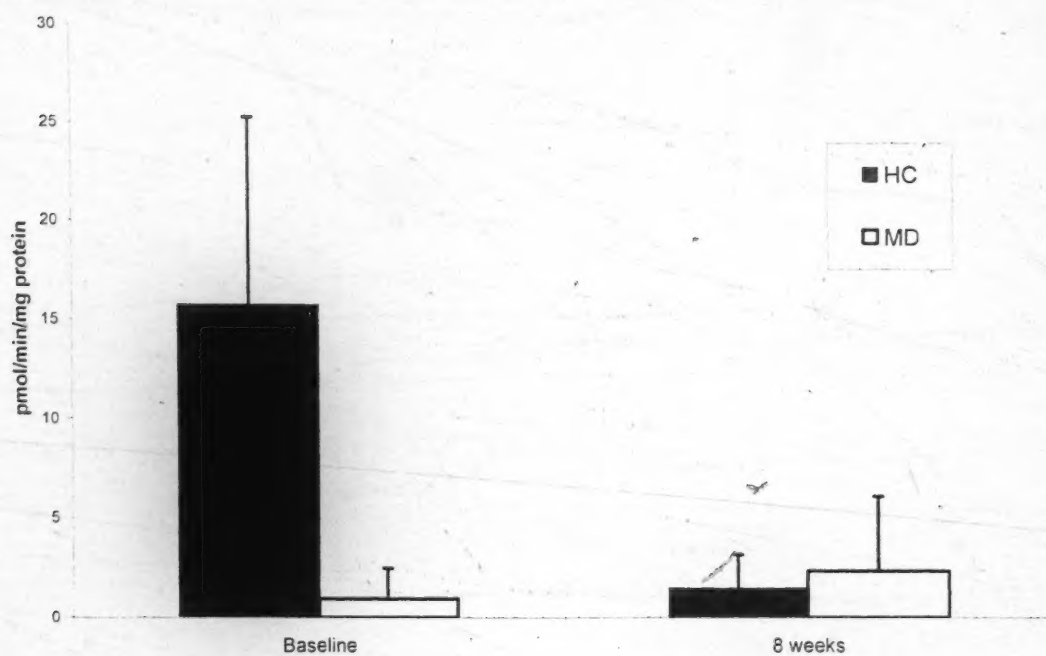


Figure 3-2. Platelet nitric oxide synthase activity in major depressive patients compared to healthy controls before and after 8 weeks of treatment with paroxetine

Legend for Figure 3-2. Values are means $\pm$ standard deviation.

TABLE 2.					
Patient Characteristic	MD	HC	t value	df	p value
Physical Activity (kcal/kg/day)	35.04 ($\pm$ 3.43)	35.45 ($\pm$ 1.99)	0.37	27	0.72
LDL-Cholesterol (mg/L)	120.96 ($\pm$ 44.26)	102.73 ($\pm$ 34.89)	-1.19	27	0.25
HDL - Cholesterol (mg/L)	43.96 ($\pm$ 11.13)	48.52 ($\pm$ 7.80)	-1.22	27	0.23
Total Cholesterol (mg/L)	198.53 ($\pm$ 36.95)	171.87 ($\pm$ 31.94)	-2.02	27	0.06
Triglycerides (mg/L)	136.07 ($\pm$ 64.59)	100.87 ($\pm$ 35.71)	-1.71	27	0.10
Folate (nmol/L)	831.00 ($\pm$ 340.57)	849.17 ($\pm$ 179.80)	0.17	25	0.87
BMI (kg/m ²)	30.12 ($\pm$ 8.65)	25.92 ($\pm$ 6.75)	-1.41	27	0.17
SBP (mm Hg)	120.50 ($\pm$ 12.74)	119.36 ($\pm$ 13.15)	-0.23	25	0.82
DBP (mm Hg)	79.25 ($\pm$ 8.58)	78.64 ($\pm$ 13.24)	-0.15	25	0.89
BMI = Body Mass Index; SBP = systolic blood pressure; DBP = diastolic blood pressure; HDL = high-density lipoprotein; LDL = Low-density lipoprotein					

Table 3-2. Demographic and Laboratory Values in Study Subjects

Legend for Table 3-2. Values are expressed as mean $\pm$ standard deviation

is some overlap between subject groups for these two studies. Based on the known role of NO on endothelial function and platelet activity, these findings suggest a potential role of NO in the link between MD and CVD since both decreased endothelial function and increased platelet reactivity have been shown to be associated with MD^{4, 24}.

The current study also confirms results on the effect of paroxetine on plasma NOx levels that we reported in an independent group of male HCs⁶. The findings of that study indicated that plasma NOx levels increased significantly after treatment with paroxetine for 8 weeks, then normalised after discontinuation of the medication suggesting that paroxetine increased endothelial NO production in a reversible manner. In that study, HCs were first tested to avoid the confounding effect of depressive illness and its recovery on NOx measures, allowing the study of pharmacological, versus therapeutic, effect. The current study increased the scope of the previous studies with the assessment of the effect of paroxetine on plasma NOx levels by including female subjects and by studying a patient population to whom paroxetine is routinely administered in clinical settings. As well, multiple measures of plasma NOx levels were taken during the 8 weeks of acute paroxetine treatment to assess the chronology of paroxetine-induced increases in platelet NOx levels during paroxetine administration.

The increase in plasma NOx levels is rapid, with a statistically significant increase noted in the first 2 weeks, before the antidepressant effect takes place. Additionally, there were no differences between the MD patients who were responders compared to non-responders to anti-depressant treatment. These results suggest that the results are pharmacological in nature and are independent of the antidepressant effect of paroxetine. The hypothesis of a pure pharmacological effect of paroxetine on peripheral NO

production is also favoured by the fact that shortly after paroxetine is discontinued there is normalization of NOx levels, bringing them to the same levels as in the baseline visit. Our findings, however, do not preclude a role of brain NO in antidepressant activity.

Plasma NOx levels continued to increase over parts of the treatment period but reached a plateau in both MD patients and HCs after 6 weeks of paroxetine treatment. A longer time course of treatment would thus be necessary to assess whether this is a true plateau and whether the paroxetine induced increase in NOx plasma levels are transient or sustained.

In this exploratory study we investigated the impact of only one dose of paroxetine (20 mg/day) on plasma NOx levels. In the future, to assess a potential dose response, it would be beneficial to administer a variety of paroxetine doses, ideally against placebo, in association with measurement of plasma paroxetine concentrations at the through in order to perform meaningful correlations between plasma dose concentrations and plasma NOx levels. In the current study measurements of paroxetine plasma concentrations were only measured during the absorption phase.

In this study we investigated a paroxetine-induced increase in NOS platelet activity as a possible mechanism for the decrease in platelet activation described after treatment with paroxetine. Indeed several studies have found that paroxetine and other SSRIs decrease platelet activation whether measured directly in vivo by flow cytometry³⁸, aggregometry²⁴, or indirectly by assaying indices of platelet activation such as platelet factor 4 and β -thromboglobulin^{39, 24, 40}. Furthermore, bruising, petechiae and bleeding events have been observed in patients treated with SSRIs^{41, 42}. However, the lack of impact of paroxetine treatment on platelet NOS activity in MD patients does not lend

support to the direct effect of paroxetine on platelet NOS activity as an explanation of these previous findings. It is possible however that the paroxetine-induced in endothelium-derived NO, illustrated by the increase in NOx plasma levels, contributes to paroxetine-induced decrease in platelet activity. Indeed, endothelium-derived NO, in addition to its vasodilatory properties, also contributes to regulation of platelet activation therefore playing a role in the prevention of thrombus formation and atherosclerosis which can a trigger of acute coronary syndromes⁴³. The SSRI-induced decrease in platelet serotonin storage is likely a direct major contributor of the SSRI-induced decrease in platelet activity^{41, 44}.

The lower plasma NOx levels observed in MD patients compared to HCs suggests that the production of NO by the endothelium is decreased in MD patients. NO is an essential contributor to endothelium function and endothelial dysfunction is associated with many of the traditional cardiovascular risk factors such as hypercholesterolemia, cigarette smoking, hypertension, inactivity, and a high-fat diet⁴⁵. Drugs that improve outcome in atherosclerotic diseases (e.g. statins, angiotensin-converting inhibitors) also improve endothelial function^{45, 46}. Our results of paroxetine-induced increase in plasma NOx suggest that paroxetine administration may improve endothelium function in MD patients. An SSRI-induced normalization of plasma NOx in MD patients reported in the current study may have contributed to the findings in Sertraline Antidepressant Heart Attack Randomized Trial (SADHART)²². This was a prospective double-blind placebo controlled study examining the effect of treatment with the SSRI sertraline in patients hospitalised for acute myocardial infarction or unstable angina. The results of SADHART suggest that sertraline may be cardioprotective, as there were 20% fewer serious

cardiovascular events for patients randomly assigned to sertraline versus placebo. These results were not found to be statistically significant but this was likely due to lack of statistical power²².

In this study, we found that administration of paroxetine led to a 198% increase in plasma NOx levels in MD patients. By comparison, it has been shown that moderate chronic smokers display a 22% decrease in NOx plasma levels and heavy smokers a 36% decrease⁴⁷. The magnitude of the effect of paroxetine on plasma NOx levels compared to conventional cardiovascular risk factors that impact endothelium function support the clinical significance of our results.

This physiological impact of the paroxetine-induced reduction in endothelium NO production that our results suggest should be confirmed. Flow-mediated dilation of the brachial artery is one of the methods that may serve to confirm that paroxetine-induced increase in plasma NOx levels is associated with an improvement of endothelial physiology^{48, 49}.

The effect of paroxetine on increasing plasma NOx levels may not however generalize to other SSRIs since paroxetine *in vitro* is the most potent serotonin reuptake inhibitor among SSRIs. A study examining the effects of paroxetine-induced changes in NO production on cardiovascular outcomes should be conducted.

In conclusion, this study is of importance in that it further suggests a possible mechanism linking MD and CVD in the form of decreased endothelial and platelet NO production and a potential beneficial impact of antidepressant treatment in the form of paroxetine on the cardiovascular risk associated with MD.

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CHAPTER 4

General Discussion and Conclusion

4.1 GENERAL DISCUSSION AND CONCLUSION

The work reported in this thesis is the first investigation of NO measurements in unmedicated patients with MD. Our results suggest NO dysregulation in MD patients may contribute to the association between CVD and MD. They also suggest that paroxetine treatment may be cardioprotective in that it increases plasma NOx levels. In Chapter 2 plasma NOx levels and platelet NOS activity were measured in a group of MD patients compared to HCs. Decreased levels of both plasma NOx and platelet NOS activity were found in the group of MD patients.

In Chapter 3 we examine the effects of acute antidepressant treatment with the SSRI paroxetine (20 mg/day) over an 8-week period on plasma NOx levels and platelet NOS activity. There was an increase in plasma NOx levels throughout 8 weeks of paroxetine treatment in both the HCs and MD patients with return to pre-treatment levels shortly after paroxetine discontinuation. These results suggest that the effects of paroxetine on plasma NOx levels are pharmacological in nature. Paroxetine had a different effect on platelet NOS levels in HCs compared to MD patients. In MD patients paroxetine had no discernable effect on platelet NOS activity whereas it induced a very sharp decrease in platelet NOS activity in HCs. This difference may be due to the fact that platelet NOS activity was already low in MD patients at baseline.

The importance of NO in cardiovascular regulation cannot be understated. When released from the endothelium its functions include aiding in vasodilation, inhibiting proatherogenic processes, and helping to control platelet adhesion ¹. NO produced by the platelet has a very large influence on platelet function including platelet adhesion ², aggregation ³ and disaggregates preformed platelet aggregates ⁴. Alterations in NOx

levels have been found and many cardiovascular risk factors have also been associated with decreased NO production in several CVDs ^{5,6}. Our findings of decreased plasma NOx levels and platelet NOS activity are of potentially great clinical importance but direct investigation of the impact on human physiology and cardiovascular outcomes must be undertaken.

Although it remains to be thoroughly tested, several studies ^{7,8} have now suggested that SSRIs, especially high affinity SSRIs, may improve cardiovascular outcome in patients with CVD. Our findings of paroxetine-induced normalization of endothelial NO production suggest that the SSRIs effects on endothelium NO production may contribute to this suggested cardioprotective effect.

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